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A Dimerization Function in the Intrinsically Disordered N-Terminal Region of Src

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SUMMARY

The mode of regulation of Src kinases has been elucidated by crystallographic studies identifying conserved structured protein modules involved in an orderly set of intramolecular associations and ligand interactions. Despite these detailed insights, much of the complex behavior and diversity in the Src family remains unexplained. A key missing piece is the function of the unstructured N-terminal region. We report here the function of the N-terminal region in binding within a hydrophobic pocket in the kinase domain of a dimerization partner. Dimerization substantially enhances autophosphorylation and phosphorylation of selected substrates, and interfering with dimerization is disruptive to these functions. Dimerization and Y419 phosphorylation are codependent events creating a bistable switch. Given the versatility inherent in this intrinsically disordered region, its multisite phosphorylations, and its divergence within the family, the unique domain likely functions as a central signaling hub overseeing much of the activities and unique functions of Src family kinases.

In Brief

Spassov et al. report that Src exists in cells and functions as a dimer and that dimerization and autophosphorylation are codependent events. Through a comprehensive structure-function analysis, they show that the dimer is an asymmetric dimer held through the interaction of the myristoylated N-terminal unique domain of one partner with a hydrophobic pocket in the kinase domain of another.

Graphical Abstract

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AUTHOR CONTRIBUTIONS

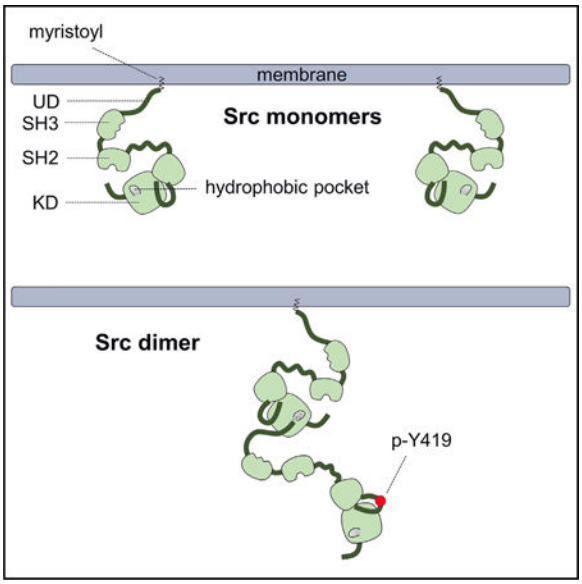
This work was conceived and designed by D.S.S., A.R.-S., and M.M.M. The experiments were performed by D.S.S., A.R.-S., and A.P. The manuscript was written by D.S.S. and M.M.M. and edited by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.09.035.



INTRODUCTION

The Src family kinases (SFKs) are a closely related family of non-receptor tyrosine kinases that play important roles in many different cellular signaling pathways (Erpel and Courtneidge, 1995; Parsons and Parsons, 2004; Thomas and Brugge, 1997). Their participation in such a wide repertoire of cellular pathways and the plethora of substrates phosphorylated by SFKs preclude the assignment of a unique function to this family, and it appears that SFKs play a fundamental role in regulating many aspects of metazoan life. The SFK family encompasses 11 members in humans by the Manning classification, including a core group of closely related members Src, Yes, Fyn, Fgr, Blk, Hck, Lck, and Lyn and a more distantly related group of Frk, Srm, and Brk (Manning et al., 2002). Src, Yes, and Fyn are widely ex-pressed in most tissue types, whereas the other members have a much more restricted expression pattern. All SFKs share a simple modular domain structure consisting of SH3, SH2, and catalytic kinase domains (KDs) followed by a short C-terminal tail, all of which are highly homologous within the family. However, N-terminal sequences of ~70–80 residues are entirely divergent imparting a unique identity and possibly unique and nonredundant function to individual members. The more closely related core SFK members also share a few residues at the extreme N terminus following cleavage of the starting methionine, providing a recognition motif for myristoylation of the N-terminal glycine by N-myristoyl transferase (NMT) (Resh, 1994, 1999). This motif, along with several charged amino acids within the first 10–17 residues that enhance affinity to membrane lipids, is referred to as the SH4 domain and accounts for the localization of these SFKs to membranes (McCabe and Berthiaume, 1999; Resh, 1994).

Decades of efforts have revealed considerable insights into how the activities of SFKs are regulated by their modular structure (extensively reviewed in Boggon and Eck, 2004; Roskoski, 2004; Sicheri and Kuriyan, 1997; Xu et al., 1999). Key determinants of their activity are phosphorylation and protein-protein interactions. Phosphorylation at Tyr 527

(using chicken c-Src numbering) by C-terminal Src kinase (CSK) is inhibitory, while phosphorylation at Tyr 416 is activating, although neither of these phosphorylations by themselves exert full positive or negative regulatory control. Pioneering crystallographic studies reveal that Src is held in an autoinhibited state by intramolecular interactions wherein the SH2 domain is bound to its phosphorylated C-terminal Tyr 527 and the SH3 domain engages a proline-rich SH2-KD linker region and the kinase N-lobe. In this state, the active site of the KD is disrupted by displacement of the aC helix. These intramolecular interactions are weak in nature and can be destabilized by solicitation of the SH2 and SH3 domains by higher-affinity ligands (Moarefi et al., 1997) or by dephosphorylation of Tyr 527 by a number of phosphatases (Roskoski, 2005). Auto-phosphorylation at Tyr 416 stabilizes the activation loop, an event that in most kinases is required for catalytic activity. Thus, the selection of substrates by Src is directly influenced by ligand recognition via the SH3 and SH2 domains and not simply by the substrate motif preferences of its catalytic domain active site in what has been termed the “turned on by touch” mode of signaling (Boggon and Eck, 2004). As such, the activities of SFKs are greatly affected by their molecular surroundings. These structural modules are highly homologous across the entire family, leaving unanswered why the family is so large and how individual members can perform unique functions.

A key feature of SFKs that is missing in current models of their regulation is their unique domains (UDs). This is because the UD is intrinsically disordered and has eluded analysis in the crystallographic studies that have informed much of our current understanding of SFK regulation. However, considerable lines of evidence suggest that the UD is critically involved in the regulation of SFKs. Although the UD sequences are not conserved between family members, they are well conserved across species, suggesting that it is not merely a spacer region but rather harbors specific functions. Furthermore, although there is little sequence homology between the UDs of SFKs, studies using nuclear magnetic resonance (NMR) and small-angle X-ray scattering reveal compaction of the UD mediated through several long-range interactions between aromatic residues that are conserved within the SFK family, suggesting that these disordered regions share at least some common structural features (Arbesú et al., 2017). The UDs of individual SFK members confer specific signaling functions distinct from other members, and in fact some of these functions can be lost by UD deletion or transferred by UD-swapping experiments (Carrera et al., 1995; Hoey et al., 2000; Summy et al., 2003; Wer-dich and Penn, 2005). Further consistent with a regulatory function, the UDs of SFKs are phosphorylated and dephosphorylated on several sites, affecting their signaling functions (Amata et al., 2013, 2014; Hansen et al., 1997; Johnson et al., 2000; Joung et al., 1995). How these phosphorylations regulate function remain largely unknown, awaiting more mechanistic insights into the functions of the UD. The most detailed function described thus far is for the UD of Lck. Lck binds the T cell co-receptors CD4 and CD8 on the surface of T lymphocytes through its UD (Shaw et al., 1989). Although its UD is unstructured in isolation, it adopts an organized heterodimeric solution structure when complexed with zinc and the cytoplasmic tails of CD4 or CD8, providing a glimpse of how this disordered region can mediate a highly specific interaction that enables Lck to function as a second messenger signaling molecule for these surface receptors (Kim

et al., 2003). Such a function has yet to be demonstrated for any other SFK UD, and in fact more recent efforts seem to imply considerable versatility in the functions of UDs.

The interest in understanding the functions of intrinsically disordered proteins (IDPs) or intrinsically disordered regions (IDRs), such as the UD of SFKs, has exploded in recent years as the sheer abundance and relevance of IDPs and IDRs in the eukaryotic proteome has become apparent (Dunker et al., 2002; Wright and Dyson, 1999, 2015). IDPs frequently exhibit multiple interaction capabilities and function as protein interaction hubs in cell-signaling networks (Dunker et al., 2005; Kim et al., 2008). Although intrinsically disordered, they often contain small elements that fold upon binding to protein targets, mediating interactions characterized by high specificity but modest affinity. Such interactions begin and end with rapid kinetics and thus are highly dynamic and often transient (Dyson and Wright, 2005; Wright and Dyson, 1999). The modest affinities of IDPs allows for a substantial degree of regulation through post-translational modifications, enabling them to function as signaling hubs (Borg et al., 2007; Gsponer and Babu, 2009; Van Roey et al., 2012, 2013). The emerging evidence and the developing paradigms for how IDRs can mediate key regulatory functions has renewed our interest in understanding the functions of the disordered UDs at the N-terminal region of SFKs.

In this work, we began by asking whether SFKs signal as dimers, what structural elements may be mediating the formation of such dimers, and how dimerization affects signaling function. The evidence implicates the UD along with the N-terminal myristoylation as critical mediators of dimerization and highly relevant for kinase activity and substrate phosphorylation.

RESULTS

Src Forms Dimers Involving the SH4-UD and KD Regions

Dimerization is a key mechanism mediating the signaling functions of many protein kinases. However, a role for dimerization has not been described for SFKs. We began this study by asking whether Src dimerizes, and if so, to determine the structural determinants that mediate dimerization and the potential relevance of dimerization in Src signaling. Constructs were generated using the full-length human c-Src or various deletion mutants lacking one or more of its functional domains. The structures and nomenclature used for all these constructs are schematically depicted in Figure 1. All constructs were generated in duplicate with either a hemagglutinin (HA) tag or a FLAG tag at the C terminus, enabling simple detection of dimerization through HA or FLAG co-immunoprecipitation assays. Transient transfection of these constructs was done in SYF cells (Src-Yes-Fyn null), which lack the expression of the SFK family in order to eliminate any background effects related to competition from endogenously expressed SFK proteins. The transfection efficiencies are high and the expression levels achieved in these experiments are roughly comparable to the endogenous expression of Src in many human cell lines, and thus the interactions detected in these studies are not due to artifactually high expression (Figure S1). The high transfection efficiency of GFP and full-length Src provides a reasonable estimate of this transfection technique in this cell type, although there may be differences in efficiency among the many different mutant constructs used.

From these HA or FLAG co-immunoprecipitation assays, it is clear that Src forms dimers (Figure 2A). Dimerization of Src requires the N-terminal region containing the SH4 and UD (Figure 2B, lane 3 versus lane 7; Figure 2C, lane 1 versus lane 2). Dimerization is maintained in constructs lacking the SH3-SH2 domains (Figure 2B, lane 3). Dimerization is greatly impaired, although not completely lost, in constructs lacking the UD and SH3-SH2 domains but maintaining the SH4 domain (Figure 2B, lane 5). Dimerization is also greatly impaired, although not completely lost, in constructs lacking myristoylation due to G2A mutation (Figure 2C, lane 3). The SH3-SH2 domains have no ability to dimerize with themselves (Figure 2D, lane 5) and are dispensable for dimerization of full-length Src (Figure 2B, lane 3). The KD has no ability to dimerize with itself (Figure 2B, lane 7); however, the KD is not dispensable for the dimerization of Src, and constructs lacking the KD are impaired in dimerization (Figure 2D, lane 3). The N-terminal SH4-UD segment is not able to dimerize with itself. This cannot be demonstrated through the simple expression of an SH4-UD segment, since this instrinsically disordered segment by itself is unstable and very poorly expressed (Figure S2). However, the SH4-UD region fused to mCherry is stable and expressed and shows no detectable self-dimerization (Figure 2E). Taken together, these data suggest that the N-terminal SH4-UD region and the KD region are the functional determinants of dimerization. Both myristoylation and the adjacent UD region have functions that contribute to the dimerization of Src. This is evident in assays using constructs that lack only the UD domain or constructs that are defective in myristoylation due to mutation of the myristoylated glycine 2 residue. In these assays, separate deletion of myristoylation or the UD region partially, but not completely, diminishes dimerization (Figures 2B and 2C).

Dimerization Is Asymmetric

The assays described above query the dimerization of identical constructs. In experimental designs that query the dimerization of nonidentical constructs, it is evident that the N-terminal SH4-UD is only required on one partner for dimerization (Figure 2G, lane 2). A partial decrease in dimerization is evident when the SH4-UD region is present in only one partner. On the other hand, the deletion of the KD in either partner prevents dimerization (Figure 2H, lanes 2 and 3). This implicates a more complex role for the KD, which is explored further below.

Dimerization Is Direct

The observed interaction of Src proteins is a direct interaction and not a molecular proximation afforded by larger cellular macromolecular protein complexes. This is evident when recombinant purified HA-tagged and FLAG-tagged Src proteins are incubated by themselves *in vitro* (Figure 2I). Purified Src run on a native gel migrates in two molecular weight forms consistent with monomers and dimers (Figure 2J). The relative ratio of monomers to dimers in this assay is likely not a faithful reflection of in-cell dimerization due to dimer disruption by the stringent purification process. Larger oligomeric forms may also be potentially disrupted during purification, and the macromolecular environment of Src in cells may involve larger forms. The cell-based assays suggest that the minimal regions necessary for dimerization are the N-terminal region and the KD. The interaction of these two domains can also be recapitulated *in vitro*. While the N-terminal SH4-UD region cannot

be expressed and purified by itself, it is stable and can be purified when fused with glutathione S-transferase (GST). This recombinant purified SH4-UD-GST protein binds a purified KD, confirming the affinity of the N-terminal region of Src with the C-terminal KD of Src (Figures 2K).

Src Dimerization Is Directly Evident in Living Cells

The dimerization of Src observed in co-immunoprecipitation assays is an in-cell interaction and not a post-lysis artifact. For more direct evidence of Src dimerization in living cells, we took an orthogonal approach using a protein fragment complementation assay. The complementation of fragmented SNAP tags is particularly useful to detect stable protein-protein interactions, as it is not complicated by the irreversible complementation seen with many split fluorescent proteins (Mie et al., 2016). The signal generated by the complementation of split SNAP tags fused to the C terminus of Src provides direct evidence in living cells of Src dimerization (Figure 3). The dimer-ization observed in this assay requires the N-terminal SH4-UD of Src, consistent with the biochemical studies discussed above (Figure 3).

Y419 Phosphorylation and Open Conformation Are Required for Src Dimerization

The dimerization of Src is linked with its activation state and conformational dynamics. Dimerization is promoted by the open active state of Src, and the closed tethered state is nonpermissive to dimerization. This is evident in dimerization assays using the Y530F mutant that promotes the open state by destabilizing the Y530-SH2 interaction (Liu and Pawson, 1994) or using the 530YEEI mutant that promotes the closed state by stabilizing the Y530-SH2 interaction (Schindler et al., 1999) (Figure 4A). Auto-phosphorylation of Y419 is required for dimerization, as evident in Y419F mutation analysis (Figure 4B). This is also evident in kinase-dead constructs that cannot auto-phosphorylate and thereby fail to dimerize (Figure 4C). Experiments with kinase inhibitors shed more subtle insights into the requirement for autophosphorylation in dimerization. If Src inhibitor treatment is applied immediately upon Src expression, then dimers will not form (Figure 4D, lane 2). However, if Src is inactivated long after its expression and dimerization, then preformed dimers are not disrupted despite clear dephosphorylation of Y419 (Figure 4D, lane 3; Figure S3). The observed reduction of dimerization following 1 hr of late Src inhibitor exposure is not due to disruption of preformed dimers; rather, it is due to the natural turnover of Src and the inhibition of dimerization in newly expressed Src molecules. This becomes most evident if the late initiation of Src inhibitor treatment is allowed to continue for a prolonged time frame beyond the life-time of Src proteins that may have been previously engaged in dimers (Figure 4D, lane 4). These experiments are consistent with the scenario wherein autophosphorylation of Y419 is a prerequisite for dimerization but is thereafter dispensable once the dimer is formed.

Y419 Phosphorylation and the SH4-UD Are Required in *cis*

Assay designs querying the dimerization of nonidentical constructs provide more clarity regarding the requirement for kinase activity and autophosphorylation in dimerization. Autophosphorylation at Y419 is required only in one partner for dimerization (Figure 4E, lanes 6 and 7). Since the N-terminal region was also shown to be required in only one

partner, a logical next question is whether these two requirements are in *cis* or in *trans*. Comparing dimerization in a variety of experimental arms designed to test whether the N-terminal region and the Y419 autophosphorylation are required in *cis* or in *trans*, it is evident that this requirement is in *cis* (Figure 4E, lane 6 versus lane 7). Similarly, an assay designed to test whether the N-terminal region and catalytic kinase activity are required in *cis* or in *trans* revealed that this requirement is also in *cis* (Figure 4F, lane 3 versus 4). Taken together, this means that for dimerization to occur, partner A must contain an SH4-UD region and be auto-phosphorylated on its Y419 in order to dimerize with partner B. The autophosphorylation of Y419 is an intermolecular phosphorylation involving another partner A, as Src does not have the ability for intramolecular Y419 autophosphorylation. The fact that Y419 autophosphorylation is an intermolecular event was demonstrated in a more specifically designed experiment using the K298M kinase-dead mutant of Src. Constructs were generated that contain the 20-kDa C-terminal SNAP tag. These constructs migrate higher on gels, allowing for easy identification of simultaneously expressed tagged and untagged forms of Src. In this experimental design, it is evident that the kinase-dead mutant of Src is autophosphorylated on Y419 in an inter-molecular event by a wildtype Src (Figure 4G, lanes 4 and 5).

Dimerization Involves the N-Terminal Myristate Binding to a KD Pocket in *trans*

The evidence thus far identifies the SH4-UD region as well as the KD to be determinants of Src dimerization. How these regions can mediate dimerization is not evident from our current understanding of Src structure, since the SH4-UD domain is intrinsically disordered and almost all of the X-ray crystallographic studies have been performed on constructs lacking the N-terminal region or lacking myristoylation. Studies of the closely related Abl kinase provide some insights into potential binding interfaces. Abl is myristoylated similar to Src. However, the myristoyl moiety can engage in an intramolecular interaction with the KD (Hantschel et al., 2003; Nagar et al., 2003). In this interaction, the myristoyl moiety embeds deep into a hydrophobic pocket within the base of the C-lobe of the KD (Figure S4). This engagement of the myristate group induces conformational changes in the C-lobe that affects its regulation (Nagar et al., 2003). Crystallographic studies of Src show structural features at this site of the KD that could potentially also bind myristate, and this is supported by NMR studies (Cowan-Jacob et al., 2005). In structural alignment, the overall architecture of this myristoyl pocket in Abl and Src appear nearly identical (Figure S4). To explore the possibility that this type of myristate-KD interaction may mediate the dimerization of Src, we focused on mutational studies of this hydrophobic pocket in Src (Figure 5A). Several bulkier residues lining this pocket were mutated to alanine, opening up this pocket in the KD of Src, potentially making it more permissive to myristate binding. These mutations, to varying degrees, increase the dimerization observed between the KD segment and the N-terminal region of Src (Figure 5B). On the other hand, T459 is a lone small amino acid at the gate of this pocket, and a T459F mutation introduces a bulkier residue into this site, making it more restrictive for myristate binding. Consistent with this, the T459F mutation decreases the dimerization observed between the KD and the N-terminal region of Src (Figure 5C). Taken together, these studies support the hypothesis that dimerization involves the interaction of the myristoylated N-terminal region on one partner with the KD hydrophobic pocket in another. Alternatively it remains possible that an intramolecular myristate-KD

interaction forces a conformational state that exposes another dimerization interface. To distinguish between these possibilities, the restrictive T459F mutation or the more permissive L494A mutation were introduced into the KD either in *cis* or in *trans* with the SH4-UD region. In these experiments, it is evident that the restrictive T459F mutation diminishes dimerization only when it is introduced in *trans* with the SH4-UD domain (Figure 5D, lane 2 versus lane 3), and similarly, the more permissive L494A mutation enhances dimerization only when it is introduced in *trans* with the SH4-UD domain (Figure 5E, lane 2 versus lane 3). These experiments are most consistent with a mode of dimerization wherein the N-terminal region of one partner binds to the KD of the other partner.

The binding of the myristate to the KD would be expected to come at the cost of membrane localization, since it would no longer be available for embedding into the plasma membrane. Consistent with this, the mutations that increase myristate-KD binding demonstrate a decrease in membrane localization, whereas the mutations that decrease myristate-KD binding demonstrate an increase in membrane localization (Figure S5). The myristate-KD binding is also potentially subject to competition by appropriately designed small-molecule inhibitors. Such inhibitors have been developed that bind the myristate-binding pocket in Abl (Zhang et al., 2010) (Figure S4D). This allosteric inhibitor of Abl, although not specifically designed for the analogous hydrophobic pocket in Src, does show some weak activity in increasing the membrane localization of Src, consistent with its ability to at least partially compete out the myristate-KD interaction (Figure S5D). These data are consistent with the notion that the observed dimerization of Src is mediated through myristate binding within the hydrophobic pocket of the KD analogous to Abl. Whether the UD segment also makes specific interactions with residues on the KD C-lobe is not addressed by these experiments, although numerous experiments with deletion constructs described earlier reveal that both myristoylation and the UD segments are important for dimerization.

N-Terminal Region Enhances Kinase Activity

The N-terminal region of Src is not only important in its dimerization but also contributes to kinase activity. Deletion of the N-terminal SH4-UD region reduces Src autophosphorylation (Figure 6A). This impairment of Src autophosphorylation activity is not due to increased Y530 phosphorylation (Figure 6A) and cannot be rescued by forcing the open and active conformation through Y530F mutation (Figure 6B). Both the myristoyl moiety and the UD contribute to activity with a greater impact from the loss of myristoylation. Myristoylation may directly enhance catalytic activity by its binding to the KD or alternatively may mediate an increase in substrate phosphorylation through localization effects or endorsing a specific molecular microenvironment. As an example, the catalytic activity of the Src-related cytoplasmic Abl kinase is known to be regulated by its myristoylation signal (Hantschel et al., 2003). To more directly explore the role of the N-terminal SH4-UD region in the catalytic activity of Src inde-pendent of their effects on compartmentalization, we turned to *in vitro* kinase assays. Src was expressed and purified from HEK293T cells so that it may be properly myristoylated (Figure 6C). Assay conditions were established to be in linear range with respect to duration and concentration (Figure S6). Deletion of the N-terminal SH4-UD region substantially diminishes the kinase activity of Src measured through its

autophosphorylation (Figure 6D). These are partial decreases relative to wild-type, and the elimination of myristoylation has a bigger impact than the elimination of the UD (Figures 6D, 6E, and S6). Partial differences can also be appreciated in cell-based experiments by increasing expression levels through increasing plasmid DNA amounts transfected (Figure S7). Similar results are obtained when assaying the kinase activity of Src against purified substrates, including paxillin, enolase, or Trask/CDCP1 (Figure 6F). The concentration-activity relationship of wild-type (WT) Src is consistent with an increase in specific activity as a function of concentration (Figure 6E), a finding that is consistent with a dimerization-driven mode of activation. Although it is difficult to compare soluble protein concentrations *in vitro* to compartmentalized proteins in cells, the cellular concentration of Src is estimated to be in the 1–10 nM range (Gee et al., 1986; Milo et al., 2010), and thus these *in vitro* experiments are in a concentration range that can potentially inform physiologic behavior. More-over, disruption of Src N-terminal myristoylation in several human cell lines by a selective inhibitor of N-myristoyltransferase also diminishes Src autophosphorylation and phosphorylation of the substrate FAK (Figures 6G and 6H), although in the cellular context, effects on subcellular or molecular localization may also be contributing to the observed effects.

Dimerization Is Required for Signaling Activity

Interfering with kinase activity or Y419 autophosphorylation prevents dimerization as shown by the mutational and drug inhibition studies described above. The reverse also holds true, such that interfering with dimerization interferes with the cellular phosphorylation activities of Src. This was determined by the identification and use of constructs that can interfere with dimerization in a dominant-negative manner. The Y419F-K298M-Y530F triple mutant of Src, which lacks its two main regulatory phosphorylation sites and is also kinase dead, has been used in the field as a dominant-negative version of Src. Our studies would suggest that this triple mutant exerts its dominant-negative activities by interfering with the dimerization of Src. Consistent with this notion, this triple-mutant Src, while deficient at self-dimerization, does bind with WT Src and diminishes the autophosphorylation activity of WT Src and the transphosphorylation activity of the substrate FAK (Figure 7A). Since we have shown that dimerization is mediated through the N-terminal SH4-UD region, we should be able to reproduce this dominant-negative or inhibitory activity through the expression of a competing SH4-UD segment alone. Indeed, we find through stepwise deletion of the KD and SH3-SH2 regions of this dominant-negative construct that its dominant-negative function is preserved and in fact enhanced by the smaller constructs, possibly related to higher expression of these smaller constructs (Figure 7B). The minimal SH4-UD region by itself is unstable and not expressed, but a GFP-fused version is well expressed and demonstrates that the inhibitory activity is entirely contained within this minimal region (Figure 7B, lane 5). More detailed analysis of this minimal SH4-UD-GFP construct reveals that it binds WT Src and successfully outcompetes a WT Src partner in the process, and the result is inhibition of Src Y419 autophosphorylation activity and inhibition of phosphorylation of its substrate, FAK (Figure 7C).

DISCUSSION

The experiments here provide structural and functional insights into the dimerization of Src. They show that Src does dimerize and that dimerization is mediated by the myristoylated N-terminal region of one partner and the hydrophobic pocket within the KD C-lobe of the other partner. Dimerization requires Y419 phosphorylation in *cis* with the N-terminal region but is dispensable after dimer formation. The fact that Y419 phosphorylation is required for dimerization is corroborated by Y419F mutation, kinase-dead Src mutants, and kinase inhibitor studies. But why phosphorylation of Y419 in *cis* is required for the N-terminal region to engage the KD of a partner in *trans* is not immediately clear. Stable protein interactions often involve intermediate conformational states and it is possible that the Y419 phosphorylated state of Src provides an appropriate platform for a transient conformation that precedes the more stable dimeric state. It is also possible that when Src is not phosphorylated at Y419, the N-terminal region is bound to specific proteins or engaged in a specific conformational state and that Y419 phosphorylation releases it from such a state, making it available for dimerization. Regardless of the structural basis, the functional link between Y419 phosphorylation and dimerization provides important clues regarding the role of Y419 phosphorylation in the activities of Src kinases. In the earliest days of this field, Y419 phosphorylation was thought to be integral to the activation process of Src because of reduced signaling and biologic activities observed with Y419F mutation of Src and emerging evidence from other kinases that phosphorylation in the activation loop of kinases is essential for access to the active site (Adams, 2003; Nolen et al., 2004). But numerous lines of evidence proved inconsistent with this generalization. The Y419F mutation only produces partial changes in observed activities, and these appear to be context dependent, possibly substrate dependent, discordant between *in vitro* and in-cell experiments, and not observed in all circumstances (Boerner et al., 1996; Ferracini and Brugge, 1990; Kmiecik and Shalloway, 1987). In *in vitro* kinase reactions, substrate phosphorylation occurs in competition with Y419 autophophorylation (Sun et al., 2002). Furthermore, the Y419F mutant of Src retains activity *in vitro* (Ferracini and Brugge, 1990; Piwnica-Worms et al., 1987), as does the Y419A mutant of Hck (Porter et al., 2000). In some cell signaling functions, Y419 phosphorylation appears redundant. For example, Src catalytic activity is required to phosphorylate FAK and mediate integrin signaling and cell spreading and migration, yet this integrin-induced function is performed without a detectable induction in Y419 phosphorylation of Src and is unmitigated by the Y419F mutation of Src (Cary et al., 2002). Furthermore, the *v-src* oncogene retains its tumorigenic properties despite Y416F mutation (analogous to human Y419F) (Snyder and Bishop, 1984). Cowan-Jacob et al. (2005) reported crystallographic studies of the unphosphorylated form of Src, revealing the A-loop to be in the open extended state with the active site exposed, consistent with the conformation of an active kinase. The active conformation was seen regardless of whether the kinase was bound to an inhibitor or AMP-PNP or in apo form. Although seemingly not required to stabilize its extended conformation, it was hypothesized that Y419 phosphorylation of the A-loop may facilitate binding certain substrates. It is clear now that while Y419 phosphorylation alters the biologic behavior of Src, this is not due to on or off switching of catalytic activity. Our studies establishing a functional link between Y419 phosphorylation and dimerization provide further insights into the role of Y419

phosphorylation. Our findings suggest that Y419 phosphorylation, by enabling dimerization, may promote Src to participate in specific macro-molecular complexes and thereby affect substrate access and selection by Src, affecting its functions in this more specific way rather than a nonspecific activation of catalytic function. It is possible that monomeric Src has biologic actions distinct from dimeric Src and that Y419 phosphorylation can dictate this transition. While our study establishes the existence and importance of Src dimerization, the techniques used here do not rigorously address the stoichiometry of dimerization. Future lines of study will more specifically need to determine how much of Src is involved in monomers versus dimers versus larger oligomers and establish the dynamics of these macro-molecular events using techniques more suited for these queries.

The fact that Y419 autophosphorylation is required for dimerization (Figure 4) and that dimerization is required for Y419 autophosphorylation (Figure 7) presents a seemingly difficult regulatory relationship to reconcile at first glance, since it seems to present a substantial barrier for the establishment of an autophosphorylated Src dimer. But this likely proceeds through transient unstable states of dimerization without phosphorylation or vice versa and a high threshold to adopt an initial stable phosphorylated dimer. This, in fact, describes a bistable switch common in biologic systems. While some signaling events in biological systems provided graded outputs operating like a rheostat, other signaling events in nature provide binary outputs, operating like a switch in the on or off positions. Such biological systems are stable in the off position and stable in the on position and are thus called bistable switches (Chatterjee et al., 2008; Pomerening, 2008). The barrier to activation in such bistable switches functions to ensure that the signal is not activated prematurely, and the self-perpetuating mechanism functions to ensure continuous and sustained output once activated, functioning as a sort of biology memory for an initiating signal that does not itself persist. The relationship between Src autophosphorylation and dimerization appears to describe such a bistable switch and suggests a binary signaling mode of output inherent in at least some of the functions of the Src family.

Many kinases are known to dimerize, although the modes of dimerization vary considerably between kinase families (Lavoie et al., 2014). The best-understood function of dimerization is the regulation of the on or off state of kinases through orthosteric or allosteric mechanisms. In the case of Src kinases, the observed bistable switch linked with dimerization is unlikely to correspond to catalytic on or off states. Consistent with this, and as described above, the Y419F mutant of Src is impaired in dimerization yet retains activity in *in vitro* and *in vivo* studies. More likely, the dimerization of Src affects its biologic behavior, perhaps including access to specific macromolecular complexes or engagement of specific substrates. The fact that the N-terminal region mediates dimerization when binding a partner KD yet mediates membrane localization when embedding in the plasma membrane creates a functional competition that may have additional relevance to the regulation of the activities of Src kinases. The N-terminal region is also known to mediate interactions with other proteins (Shaw et al., 1989; Vonakis et al., 1997), also potentially in competition with its dimerization function, providing yet another layer of complexity in the functions of this region. The multifunctional nature of the N-terminal domain is likely enabled by its lack of an ordered structure, allowing it to adopt different conformations during interactions with different partners. The flexibility inherent in the UD makes it an attractive candidate as a

regulatory hub that governs the overall activities of Src kinases. Adding additional layers of diversity is that fact that dimerization in the Src family is not restricted to homodimers. We also readily detect Src-Fyn heterodimers and Fyn-Fyn homodimers in similar HA or FLAG pulldown assays (not shown). Since the N-terminal UDs are divergent between Src family members, the existence of various homodimeric and heterodimeric complexes of Src kinases creates even broader diversity in potential signaling activities associated with the various homo- and heterodimers.

Our understanding of the mechanisms underlying the regulation of Src kinases was largely informed by the crystallographic studies of the late 1990s. These developments came during an era of pioneering studies revealing how structured protein modules have evolved to regulate protein function. Now, two decades later, many of the complexities in the Src family remain difficult to explain, and current models of Src regulation have major conceptual gaps that await deeper insights. These gaps may potentially be filled by a new leap in proteomics, recognizing the sheer abundance and functional significance of unstructured protein regions. Indeed, we may have underestimated the importance of the N-terminal region of Src kinases, and this unstructured region may underlie much of the complexity inherent in the functions of this kinase family. This would be consistent with an increasing body of evidence regarding the critical role of IDRs in regulating protein function. These regions can harbor structural plasticity, adopting different structures on different targets (Dyson and Wright, 2005), or they can bind via dynamically heterogeneous conformations, or they can bind without any apparent order (Baker et al., 2007; Mittag et al., 2010; Tompa and Fuxreiter, 2008; Wang et al., 2011). The conformational entropy inherent in IDRs facilitates the allosteric coupling of protein domains, placing IDRs at the heart of the processes that regulate protein functions. Since the interactions of IDRs are based on limited or transient structural features and lower-affinity interactions, they are much more affected by post-translational modifications, and it is well appreciated that IDRs are a hub for the regulation of protein function through post-translational modification (Borg et al., 2007; Gsponer and Babu, 2009; Van Roey et al., 2012, 2013). Indeed, post-translational modifications of the proteome are biased toward IDRs, including phosphorylation, glycosylation, hydroxylation, acetylation, sulfation, ADP ribosylation, SUMOylation, and ubiquitination (Iakoucheva et al., 2004; Pejaver et al., 2014). The N-terminal region of Src is no exception to this and is known to undergo multiple phosphorylations with consequent affects in its observed activities (Amata et al., 2013, 2014; Hansen et al., 1997; Johnson et al., 2000; Joung et al., 1995). The presence of other post-translational modifications of the N-terminal UD of Src has not been studied to date, but our work provides a compelling case that the functions of Src kinases may be regulated in part by post-translational modification of the N-terminal UD. IDRs such as the UD of Src can function as signaling hubs, coordinating the activities of Src kinases in response to specific modifications. Such modifications can bias the binding affinities of the N-terminal region between the KD of a dimerization partner, the plasma membrane, or an interacting protein or cytoskeletal macromolecule.

The dimerization of Src has been considered and detected by other studies but with conflicting results and no functional insights. Weijland et al. (1997) purified and studied a truncated Src protein missing the N-terminal SH4-UD region and found it to be monomeric by gel filtration chromatography, consistent with our findings using a similarly truncated

construct. Kemble and Sun (2009) studied bacterial purified Src with an interest in redox effects and reported that oxidation induces a covalent dimerization of Src *in vitro* mediated through disulfide Cys bridging. Irtegun et al. (2013) reported Y416 phosphorylation in the closed state of Src and in their studies conducted ultracentrifugation of EGFP fused Src, observing both monomeric and dimeric forms. These studies suggest the possibility that Src may form dimers, but the lack of myristoylation, the presence of dimerizing tags, or the lack of in cell evidence has not allowed confirmation and insightful analysis of dimerization. Le Roux et al. (2016a, 2016b) studied the kinetics of *in vitro* liposome binding to N-terminal Src regions and reported that the myristoylated SH4 region alone binds with kinetics that suggest dimerization is involved. The near-complete lack of protein content in this minimal construct and its dependency on lipid composition in the liposome may indicate the role of this region in the clustering of Src at the membrane and in membrane microdomains rather than dimerization involving protein interfaces. The arrangement of Src in some packed crystal structures of KD fragments has been interpreted as dimeric (Breitenlechner et al., 2005), but the relevance of these crystal findings to the physiologic structure of the full-length native protein is doubtful.

The importance of Src myristoylation has been known for some time, but this has largely been attributed to its role in mem-brane localization. Src constructs defective in myristoylation have altered phosphorylation activities (Bagrodia et al., 1993; Linder and Burr, 1988) and loss of transforming ability (Kamps et al., 1985, 1986), but the functions of the myristoyl moiety exceed beyond simple membrane localization. In some proteins, myristate is known to bind intramolecularly within hydrophobic pockets of the protein. In these scenarios, alternative binding modes (i.e., to the membrane versus the protein) affect protein function and define a myristoyl switch, such as seen in HIV-1 gag, recoverin, and Abl (Ames et al., 1995, 1996; Hantschel et al., 2003; Nagar et al., 2003; Resh, 2004; Tanaka et al., 1995). Src may also be regulated by a myristoyl switch, and our results support this model, but structural evidence for such a binding mode has thus far eluded crystallographic studies. Patwardhan and Resh (2010) conducted a biochemical analysis of myristoylated and non-myristoylated Src and reported effects on kinase activity, a possible association with the KD hydrophobic pocket, and enhanced protein stability and half-life. These are partly consistent with our findings, but this study did not investigate dimerization or signaling functions. The importance of the N-terminal UD for autophosphorylation activity has been demonstrated for the more distantly SFK-related protein Srms. The N-terminal UD of this protein is required for its catalytic activity (Goel et al., 2013), although the lack of N-terminal myristoylation in Srms highlights key differences between these distantly related kinases.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-HA	SantaCruz Biotech.	F7 / sc-7392
Rabbit anti-HA	SantaCruz Biotech.	Y11 / sc-805
Rabbit Anti-Flag	Cell Signaling Tech.	cs-2368
Mouse Anti-Flag	Sigma	F1804
Anti-V5	Cell Signaling Tech	cs-13202
Anti-phosphotyrosine	SantaCruz Biotech.	PY99 / sc-7020
Anti-GST	SantaCruz Biotech.	sc-138
Anti-paxillin	Cell Signaling Tech.	cs-5574
Anti-Hsp90	SantaCruz Biotech.	sc-7947
Anti-CAV1	SantaCruz Biotech.	sc-894
Control mouse IgG	SantaCruz Biotech.	sc-2025
Anti-pY416-Src	Cell Signaling Tech.	cs-6943
Anti-pY527-Src	Cell Signaling Tech.	cs-2105
Anti-pY118-paxillin	Cell Signaling Tech.	cs-2541
Anti-Trask/CDCP1	Custom made	Custom made
Anti-mouse Alexa Flour 546	Life Technologies	A11030
Anti-rabbit Alexa Flour 647	Life Technologies	A21245
Bacterial and Virus Strains		
DH5a competent cells	Home-made electrocompetent	N/A
Chemicals, Peptides, and Recombinant Proteins		
Dasatinib	EMD-Calbiochem	discontinued
Precision Protease	GE Healthcare	27–0843-01
Glutathione Sepharose beads	GE Healthcare	17–0756-01
Protein G Sepharose beads	GE Healthcare	17–0618-02
SNAP cell Oregon Green	New England Biolabs	59104
Hoechst	ThermoFisher Scient.	62249
Paxillin	Raybiotech	230–00595-10
Enolase	Sino Biological Inc	11554-H07E
Critical Commercial Assays		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BP Clonase II	ThermoFisher Scient	11789100
LR Clonase II	ThermoFisher Scient	11791100
Kinase Assay buffer II	SignalChem	K02–09
Mem-Per Membrane Protein Extraction Kit	ThermoFisher Scient	89842
NativePage novex bis-Tris gels	Life Technologies	BN1002BOX
Experimental Models: Cell Lines		
SYF cells	ATCC	CRL-2459
HEK293T cells	ATCC	CRL-3216
MDA-MB-468 cells	ATCC	HTB-132
MDA-MB-231 cells	ATCC	HTB-26
KM20 cells	Gift of Dr. Michael Korn	N/A
WiDr cells	Gift of Dr. Michael Korn	N/A
Oligonucleotides		
Extensive primer sequences provided in Table S1		N/A
pDONR221	ThermoFisher Scient	12536017
pDEST40	ThermoFisher Scient	12274015
pDEST40-2XHA	Modified from pDEST40	N/A
pDEST40-2XFLAG	Modified from pDEST40	N/A
pDEST40-PP-GST	Modified from pDEST40	N/A
pDEST40-2XHA-SNAP	Modified from pDEST40	N/A
pDEST40-2XFLAG-SNAP	Modified from pDEST40	N/A
pDEST40-2XHA-nSNAP	Modified from pDEST40	N/A
pDEST40-2XFLAG-cSNAP	Modified from pDEST40	N/A
pCMV6-cSRC	Origene	sc125208
Software and Algorithms		
Photoshop	Adobe	N/A
Powerpoint	Microsoft	N/A
Excel	Microsoft	N/A
Prism	Graphpad	N/A
Pymol	Open source	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ImageJ	NIH	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for information and reagents should be directed to and will be fulfilled by the Lead Contact, Mark M. Moasser at the University of California, San Francisco Helen Diller Family Comprehensive Cancer Center, mark.moasser@ucsf.edu.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

SYF, HEK293T, MDA_MB-468, and MDA-MB-231 cell lines were obtained from the American Type Culture Collection and grown in DMEM/F12 medium containing 10% fetal bovine serum, and 1% (v/v) *penicillin*–streptomycin solution. KM20 and WiDr cells were a gift from Dr. Michael Korn and were grown in the same medium.

METHOD DETAILS

Plasmid constructs—The cloning strategy was based on the Gateway system (ThermoFisher Scientific). The pDEST40 vector was modified to have either 2xHA or 2xFLAG tags in-frame at the C terminus of the inserts. The mutant constructs were generated in pDONR221 vectors and subsequently shuttled into both pDEST40-2XHA and pDEST40-2XFLAG destination vectors. In the constructs designed for protein purification, the V5, HA, or FLAG tags were fused in frame with the carboxyl terminus, followed by a PreScission protease cleavage site and an in-frame GST (pDEST40-PP-GST).

The coding sequence of human c-Src was amplified from c-Src cDNA clone sc125208 (Origene). The primer sequences for this and all other construct cloning and mutation are provided in Table S1. Some constructs were cloned by amplification of the desired segment with appropriate primer extensions for cloning into pDONR221. The amplified PCR product was recombined by using BP Clonase II (ThermoFisher Scientific) into pDonR221. Some constructs were made by deletion cloning, i.e., outward PCR amplification of the entire plasmid omitting the desired deletion region. These were done by low number amplification cycles using Pfu Ultra HF polymerase and 5^0 phosphorylated primers followed by a self ligation reaction and DpnI digestion. Point mutations were created by site-directed mutagenesis using Pfu Ultra HF polymerase, whole plasmid amplification, followed by DpnI digestion and ligation in bacteria. All constructs were sequenced across the insert to verify the identity of the construct and rule out the presence of undesired mutations. The constructs in pDONR221 were moved to destination vectors using LR Clonase II (ThermoFisher).

Purification of src proteins—Plasmids containing the desired WT or mutant Src constructs with in-frame C-terminal tags (V5, HA,FLAG) followed by a PreScission protease cleavage site and GST were transfected in HEK293T cells (typically 40–60 μg plasmid per 15 cm dish). Next day the cells were treated with 1mM dasatinib (unless otherwise stated) for 1 hour to dephosphorylate Src and cells lysed in HEK293T cells lysis buffer (20mM Tris HCL, pH 7.5, 1% Triton X-100, 10% Glycerol, 400 mM NaCl, 1mM EDTA, 200 nM DTT, 1 mM PMSF, 2 μg/ml Leupeptin, 2 μg/ml Aprotinin, 1 nM sodium vanadate). The lysates were incubated at 4ºC for 30 minutes with constant rotation, and then centrifuged at 16,000 g for 5 min. The supernatant was collected and incubated with Glutathione Sepharose 4B beads (GE Healthcare)at 4ºC for 3 hours with constant rotation. The beads were washed 4 times with HEK293T lysis buffer and once with PPCB (PreScission Protease Cleavage Buffer: 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), pH 7.0). Next the beads were incubated with 1U PreScission protease (GE Healthcare) in PPCB at 4ºC overnight with constant rotation. Next day the supernatant, containing the purified src proteins was carefully collected. Typically, around 10% of the sample was run in a gel for Coumassie staining. The purified proteins were stored at −20ºC in 50% glycerol.

Split SNAP-tag complementation assay—The split SNAP-tag complementation assay was performed by splitting the SNAP tag into nSNAP and cSNAP fragments following Gln91 according to a previously established complementation assay for living cells (Mie et al., 2012). Modified pDEST40 vectors were engineered with c-terminal nSNAP or cSNAP tags following 2XHA or 2XFLAG tags to generate pDEST40-2XHA-nSNAP and pDEST40-2XFLAG-cSNAP expression vectors. All constructs were confirmed by sequencing. WT and mutant Src inserts were then cloned into these vectors. These vectors were transfected into SYF cells using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). 24h after transfection, cells were labeled with SNAP-Cell Oregon Green (1 uM) for 30min. The cells were washed three times to remove unreacted substrate, counterstained with 5uM Hoescht 33342 for 2 minutes and incubated in fresh medium for 30 min. Cells were fixed with 4% formaldehyde solution (Thermo Scientific) and

permeabilized with 0.02% Triton X-100. Cells were blocked and stained with the indicated anti-HA antibody (Y-11 sc-805 Rb) at 1:500 and anti-FLAG Ab (Sigma F1804 Ms) at 1:200 followed by the appropriate secondary antibodies conjugated with Alexa Fluor 546 (excitation at 488 nm and emission at 585–615 nm) or Alexa Fluor 647 (excitation at 633 nm and emission from 650 nm). Cells were imaged using a Spinning disk confocal Nikon TI inverted microscope (× 40 objective, lasers 405, 488, 561 and 648 nm). For each transfection, at least 49 cells were analyzed using ImageJ. The complementation signal was plotted as a function of expression levels of the individual constructs.

Immunoprecipitation assays—Equal amounts (250ng-2 μg) of indicated HA and FLAG–tagged src constructs were co-transfected in SYF cells (6cm or 10 cm dishes). Total cellular lysates were harvested in modified RIPA buffer (10 mM Na phosphate (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% NP40, 1% Na deoxycholate, protease inhibitors and 1mM sodium orthovanadate). For immunoprecipitation studies, 200–300ug of lysate in mRIPA was incubated overnight with mouse anti- HA antibodies (clone F7, SantaCruz Biotechnology), immune complexes collected by protein G-Sepharose beads (GE Healthcare), washed, and the denatured complexes separated by SDS-PAGE, transferred to membranes, and immunoblotted with appropriate antibodies. For western blotting, 30ug of each lysate was separated by SDS-PAGE, transferred to membrane, and immunoblotted using appropriate primary and secondary antibodies and enhanced chemoluminescence visualization.

For detection of Src dimers using native gel electrophoresis, Src and SrcG2A constructs were purified from HEK293T cells as described above. Equal amounts of purified Src and SrcG2A were loaded into nativePAGE Novex Bis-Tris gels (Life Technologies). Electrophoresis was performed in the presence of Coomassie G-250 in the cathode buffer according to the manufacturer's instructions (#BN1002BOX Life Technologies). Gels were transferred onto a PVDF membrane and blocked with 3% bovine serum albumin. The membranes were stained with primary antibodies overnight at 4ºC, washed 3 times with TBST (Tris-buffered saline containing 0.5% tween), stained with secondary antibodies for 1 hour at room temperature and detected using the chemiluminescence method.

***In vitro* interaction of purified S4-UD with KD—**For purification of the N-terminal S4-UD region fused to GST, the construct did not contain the Precision Protease cleavage site. The S4-UD-GST fusion construct was expressed in HEK293T cells and lysates loaded on Glutathione Sepharose 4B beads (GE Healthcare) at 4ºC for 3 hours and subsequently washed 4 times with HEK293T lysis buffer and once with mRIPA. To demon-strate the interaction with the KD, the purified Src KD was incubated with the S4-UD-GST bound beads at 4ºC overnight in mRIPA buffer. Next day the bound proteins were eluded with Elution Buffer (25 mM glutathione, 50 mM Tris, pH 8.8, 10 mM DTT, 200 mM NaCl, 10% glycerol)) and boiled in Laemmli buffer. The proteins were separated by SDS-PAGE and immunoblotted.

***In vitro* dimerization of purified src proteins—**HA and FLAG tagged src constructs containing Y530F mutation were purified from HEK293T cells as described above, but the cells were not treated with dasatinib to preserve the endogenous Y419 phosphorylation. The src proteins were dialyzed against buffer D (50 mM Tris-HCl, 150 mM NaCl) by using

Slide-A-Lyzer MINI Dialysis Devices (ThermoFisherScientific). The dialyzed proteins were co-incubated in the presence of kinase assay buffer (see below) and immunoprecipitated with mouse anti-HA antibody, or normal mouse IgG control antibodies. The immune complexes were washed and boiled, separated on SDS-PAGE, transferred to membrane, and immunoblotted with anti-FLAG antibodies.

***In vitro* kinase assay—**The kinase assay buffer and ATP were purchased from SignalChem (Richmond, BC). The indicated amounts of purified src proteins were incubated in kinase assay buffer (5 mM MOPS, pH7.2, 2.5 mM B-glycerol-phosphate, 4 mM MgCl2, 2.5 mM MnCl2, 1 mM EGTA, 0.4 mM EDTA, 10 mM DTT, 250 mM ATP) at 30ºC for 10–30 min. The reactions were stopped with 5x Laemmli buffer and boiling for 5 min. The results were analyzed by SDS-PAGE and western blotting. Recombinant human Paxillin was purchased from Raybiotech (Norcross, GA) and recombinant Enolase (ENO3) was purchased from Sino Biological Inc.(Beijing, PRC). All recombinant proteins used as substrates were purified from bacteria and therefore were not phosphorylated prior to the kinase reactions. HEK293T cells, transfected with the desired src constructs were treated with 1 mM dasatinib (Src and Csk inhibitor) to dephosphorylate Src for 1 hour prior to lysis.

Extraction of membrane and cytosolic fractions—Separation of membrane and cytosolic fractions was performed with Mem-Per™ Plus Membrane Protein Extraction Kit (ThermoFisher Scientific). Briefly, the FLAG-tagged versions of indicated src constructs were transfected in SYF cells. Next day the cells were lysed in Permeabilization buffer and span 16,000 g for 15 min. The supernatant containing cytosolic proteins was care-fully transferred to a new tube. Any residual droplets of supernatant that still remain around the pellets were removed carefully with P10 pipette. Next the pellets were resuspended in Solubilization buffer and incubated at 4ºC for 30 minutes with constant mixing. After spinning at 16,000 × g for 15 minutes at 4ºC, the supernatants containing solubilized membrane and membrane-associated proteins were collected. The collected cytosolic and membrane proteins were run on SDS-PAGE and visualized with anti-FLAG antibodies. The efficiency of fractionation procedure was monitored with antibodies against HSP90 (localizes to the cytosolic frac-tion) and Caveolin-1 (localizes to the membrane fraction).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of image-based signals was done using ImageJ software (https://imagej.nih.gov/).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Src exists in cells as dimers
- The unique domain of one partner interacts with the kinase domain of another partner
- The myristoyl group is involved in binding a hydrophobic pocket in the kinase domain
- Dimerization and autophosphorylation are codependent events

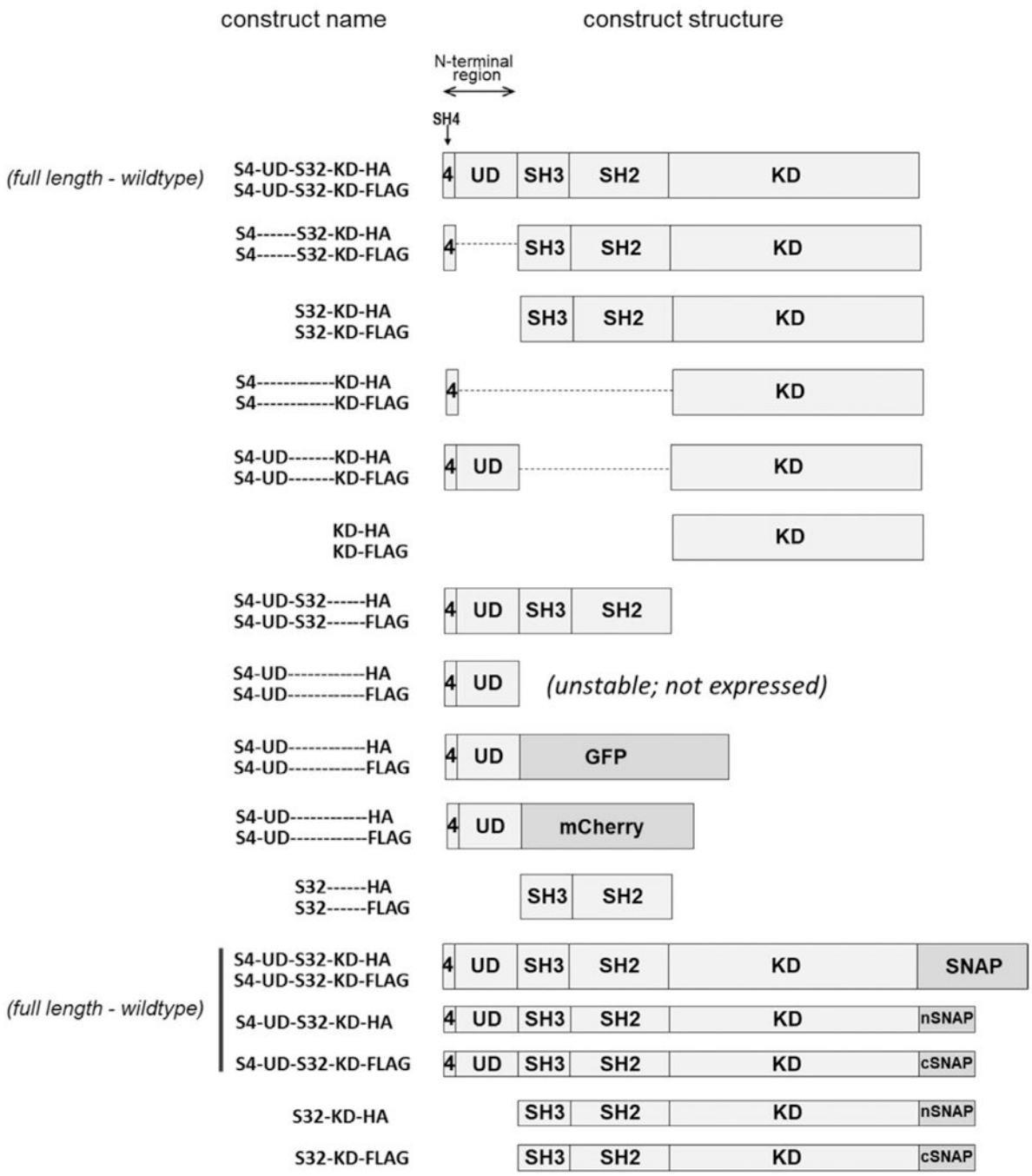

Figure 1. Index to Constructs

The various constructs designed for use in this study are shown here with the nomenclature used to refer to them. All constructs were created in both HA- and FLAG-tagged versions for easy analysis of dimerization. In addition, some of these constructs were further modified by point mutations and these are indicated in the figure labels.

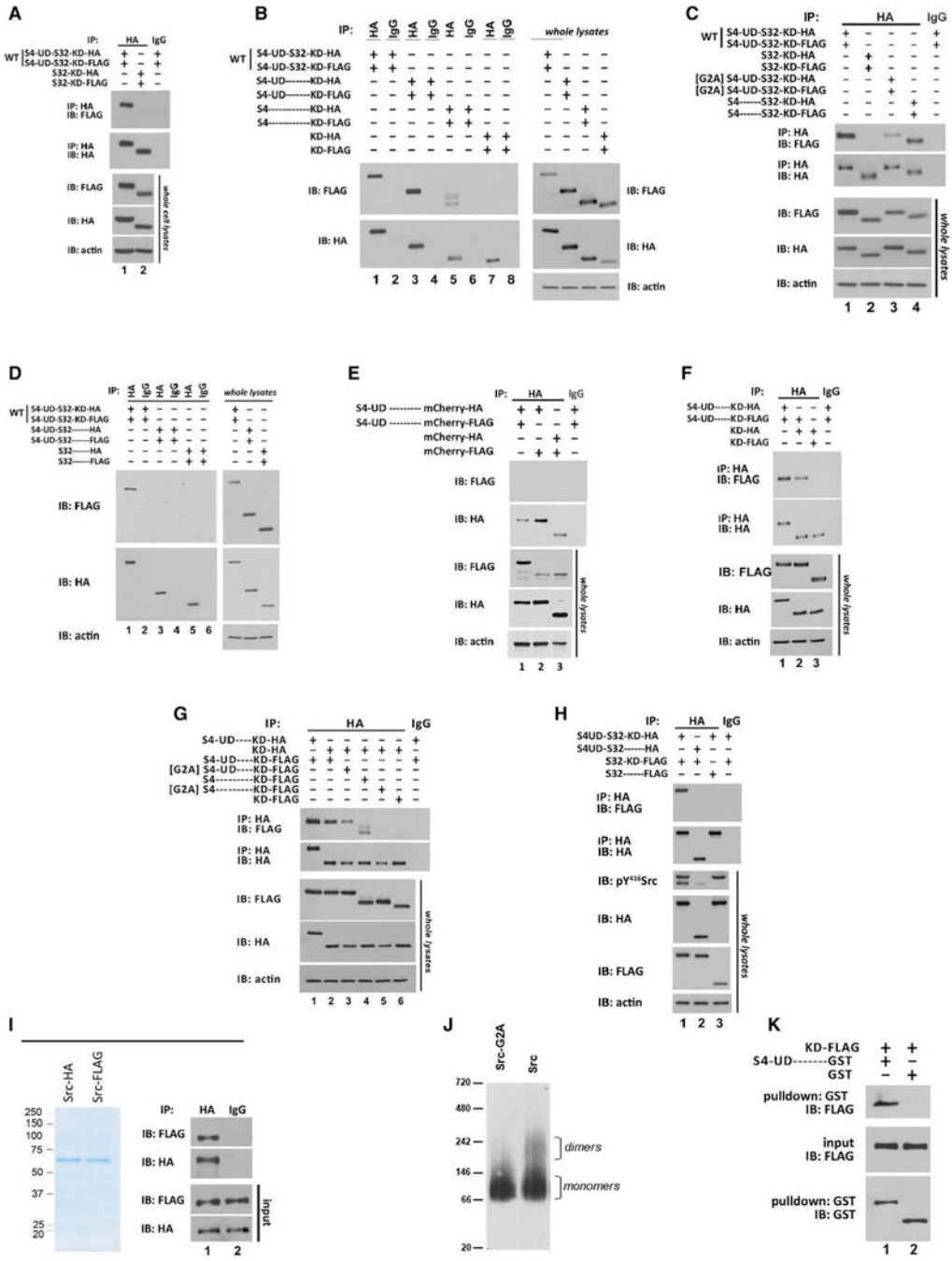

Figure 2. Dimerization of Src Requires the N-Terminal Region and Kinase Domain

(A–H) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies. Immunoblots of whole-cell lysates were also performed as indicated.

(I) Y530F mutant of Src tagged with HA or FLAG was purified from HEK293T cells. The Y530F mutant was used to best demonstrate in vitro dimerization, since Src purified from these cells has significant Y530 phosphorylation and can promote the closed conformation. Coomassie stains of the purified proteins are shown on the left. The purified proteins were

mixed and incubated for 2 hr, followed by addition of anti-HA antibodies or isotype control immunoglobulin G (IgG). The anti-HA immunoprecipitates were immunoblotted as indicated and shown on the right. A portion of the input mixture prior to immunoprecipitation was also immunoblotted as indicated.

(J) Src and SrcG2A constructs were purified from HEK293T cells and analyzed in blue native gels followed by immunoblot using anti-Src antibody.

(K) The GST-tagged S4-UD construct was expressed and purified from HEK293T cells by pull-down on glutathione Sepharose beads. The FLAG-tagged KD construct was expressed and purified from HEK293T cells by glutathione sepharose beads and the GST tag cleaved off. The purified proteins were mixed and incubated overnight at 4ºC in mRIPA buffer; beads were pulled down, eluted in GST elution buffer, separated by SDS-PAGE, and immunoblotted as indicated. Experiments with the various constructs were performed 2–4 times, and representative data are shown here.

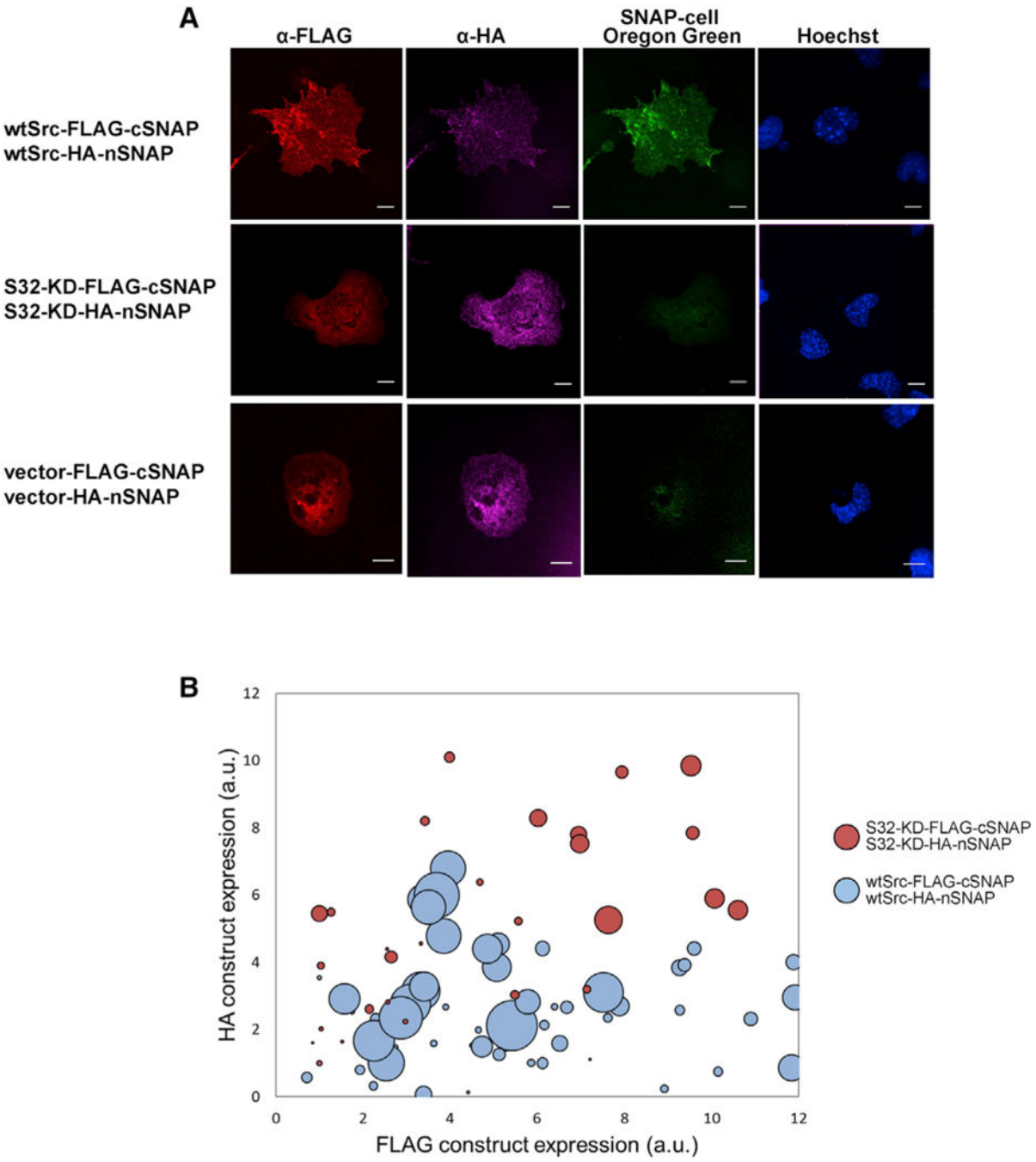

Figure 3. Dimerization of Src Is Directly Evident in Living Cells

(A) SYF cells were transfected with the indicated dual constructs carrying complementary nSNAP and cSNAP tags. The expression of each of the two constructs was evaluated by anti-HA and anti-FLAG immunostaining, and their interaction was evaluated by the SNAP-cell reagent using fluorescence reporters selected for comfortable spectral separation. Images were obtained using a spinning-disk inverted confocal microscope and a 40× objective. Scale bars, 10 μm.

(B) The two expression signals and the complementation signal were quantified in individual cells in the WT Src transfection and the S32-KD construct transfection. The expression of the two constructs (from HA and FLAG immunostains) is depicted in the *x* and *y* axes, whereas the intensity of the complementation signal is depicted in the diameter of the circles on the graph and has been corrected for background evident in vector transfected controls. Complementation of the full-length WT Src is best compared with the S32-KD construct in ranges of similar expression such as the 2–8 ranges along the *x* and *y* axes. Experiments were done 3 times, and representative data are shown here.

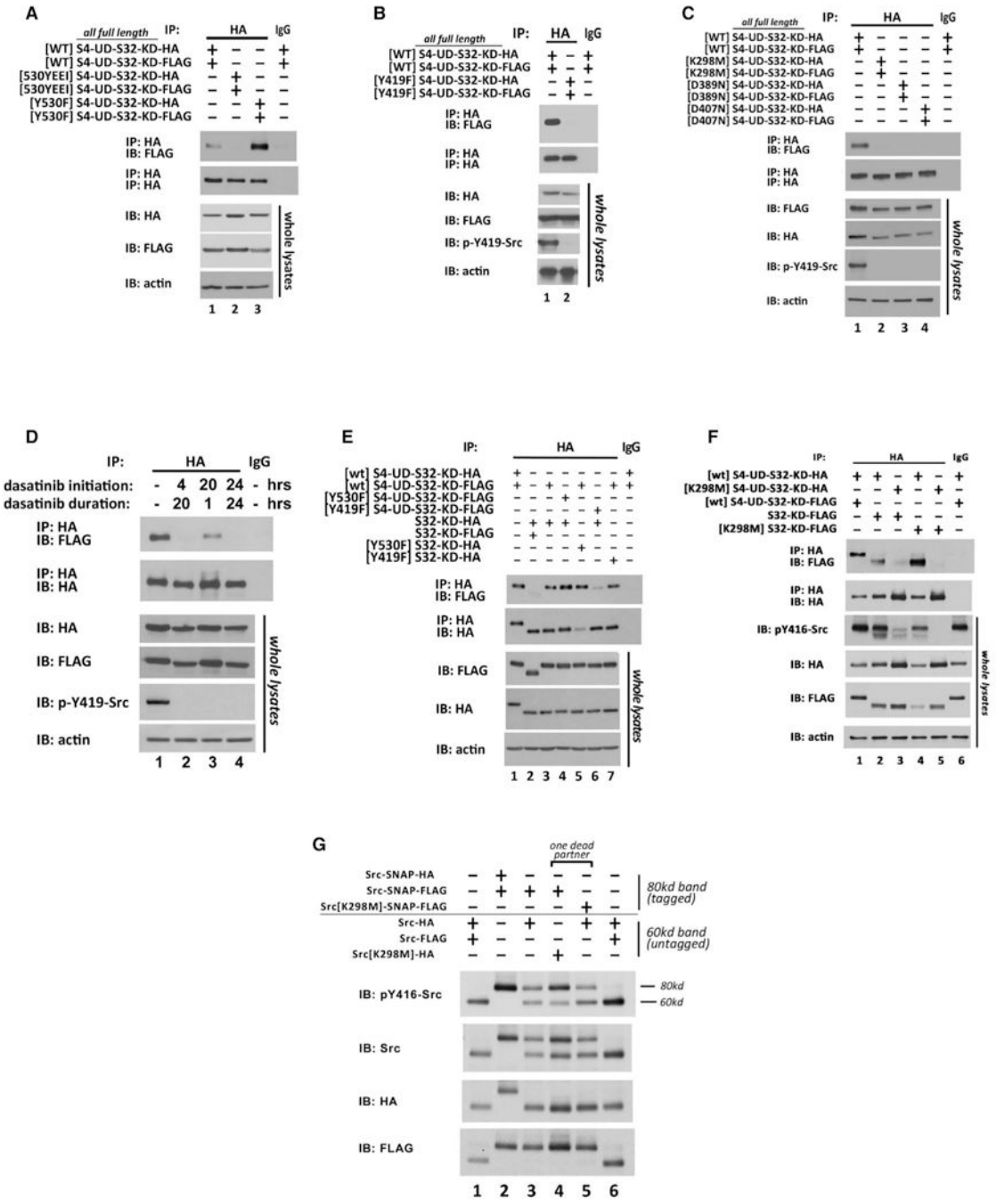

Figure 4. Dimerization of Src Requires Y419 Autophosphorylation in cis with the N-Terminal Region

(A–C) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies. Immunoblots of whole-cell lysates were also performed as indicated.

(D) SYF cells were transfected with HA-tagged and FLAG-tagged full-length wild-type Src and treated with DMSO control or 1 mM dasatinib and anti-HA immunoprecipitates were immunoblotted as indicated. The dasatinib treatment was started either early or late or for

short or long durations as indicated. For more clarity, the treatment timeline is shown graphically in Figure S3.

(E and F) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies. Immunoblots of whole-cell lysates were also performed as indicated.

(G) SYF cells were transfected with the indicated higher or lower migrating Src constructs, and cell lysates were immunoblotted as indicated. Experiments with the various constructs were performed 2–4 times, and representative data are shown here.

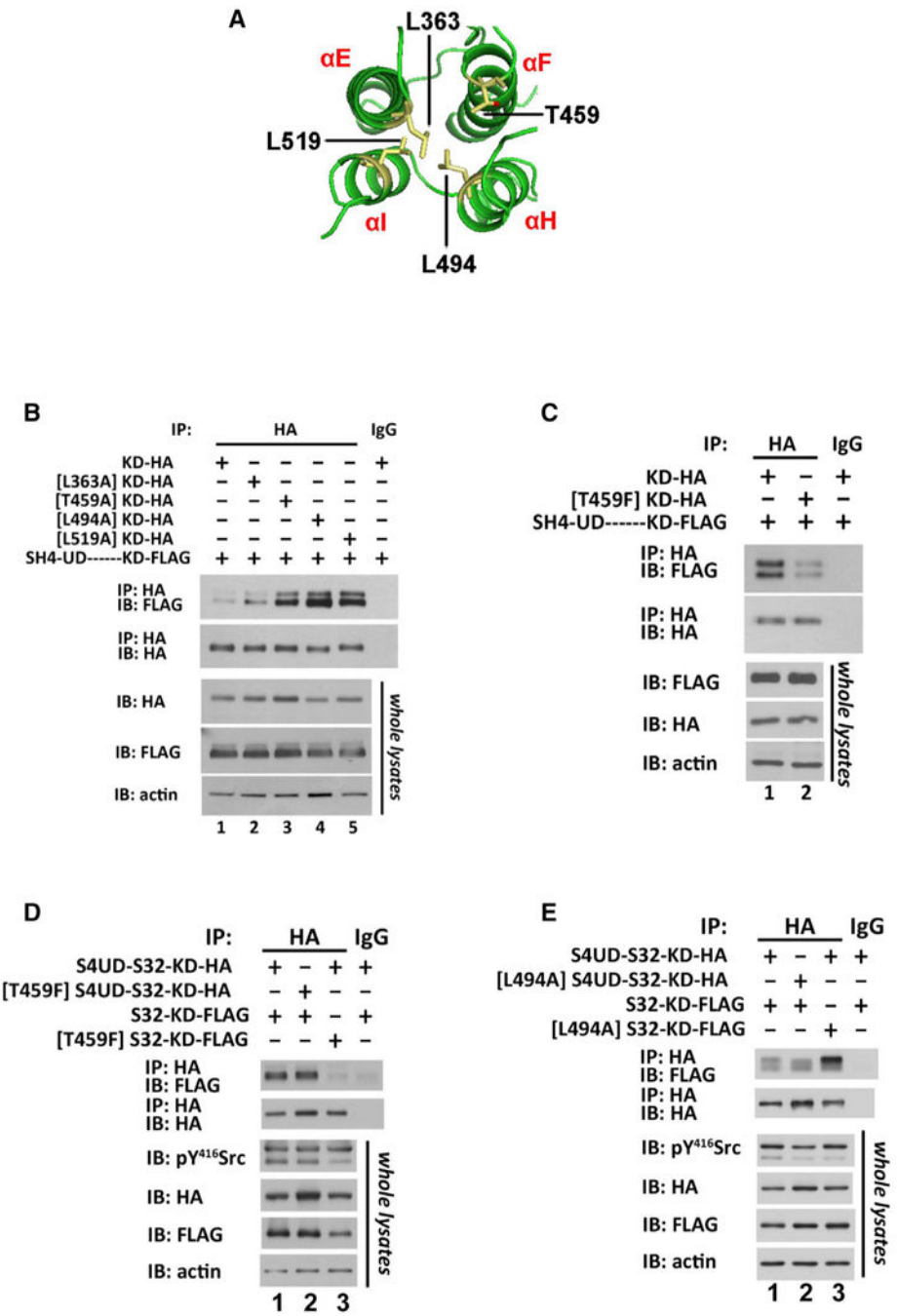

Figure 5. Dimerization of Src Is Mediated through a Kinase Domain Hydrophobic Pocket in *trans* with the N-Terminal Region

(A) The region of the putative hydrophobic pocket within the C-lobe of the Src kinase domain is shown from structure 2SRC. The residues mutated to widen or narrow the pocket are indicated.

(B–E) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies. Immunoblots of whole-cell lysates were also performed as indicated. The double bands seen in some immunoblots are due to a higher migrating Y338-phosphorylated form of Src as

determined in additional experiments. Experiments with the various constructs were performed twice, and representative data are shown here.

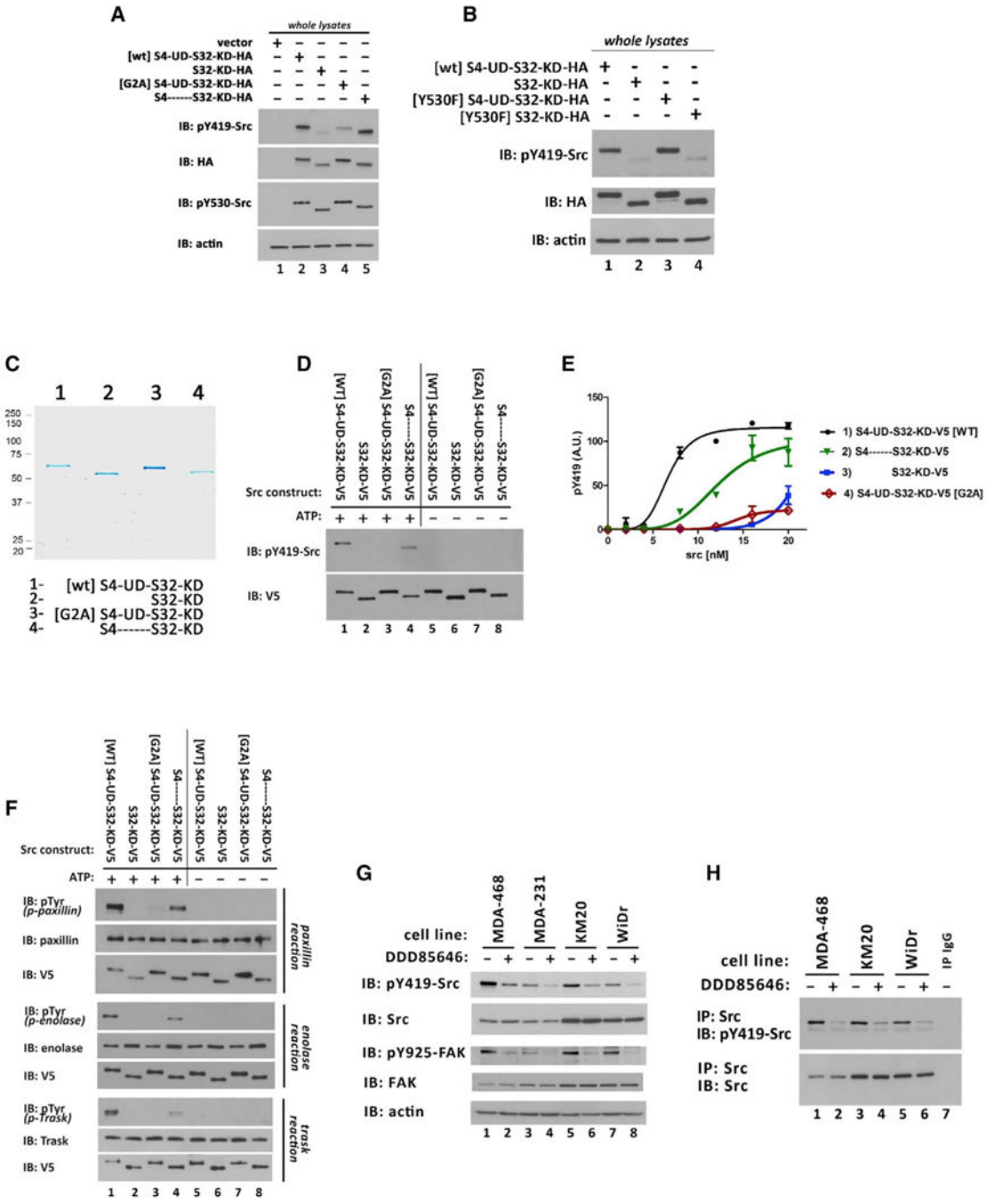

Figure 6. The N-Terminal Region of Src Regulates Catalytic Activity

(A and B) SYF cells were transfected with the indicated constructs and cell lysates immunoblotted as indicated.

(C) The indicated full-length or partial length Src constructs were expressed and purified from dasatinib-treated HEK293T cells. Dasatinib treatment was to enable the purification of unphosphorylated Src. Coomassie stains of the purified proteins are shown here.

(D) *In vitro* kinase reactions were carried with 10 nM of the indicated purified V5-tagged Src proteins for 10 min and assayed by anti-pY419Src immunoblotting. ATP was omitted in the negative control arms.

(E) The same *in vitro* kinase reactions were performed using different concentrations of protein as indicated. The quantified autophosphorylation results are shown here, and the corresponding immunoblots of these reactions are shown in Figure S6. The data points represent the average of n = 2; errors bars represent SEM.

(F) Three separate *in vitro* kinase reactions were performed using 4 nM Src proteins and each of the three indicated purified recombinant substrates. Kinase reaction linear conditions were previously established and are as follows: 40 nM paxillin in reaction for 20 min, 250 nM enolase in reaction for 20 min, and 70 nM Trask/CDCP1 in reaction for 10 min. Substrate phosphorylation was assayed by anti-pTyr immunoblotting and kinase and substrate proteins immunoblotted with V5 and substrate-specific antibodies. ATP was omitted in the negative control arms.

(G) The indicated cell lines were treated with 1 μM DDD85646 for 30 hr and cell lysates immunoblotted as indicated. Anti-pY416Src antibodies cross-react with all members of the Src family.

(H) The same lysates were assayed specifically for Src autophosphorylation by anti-Src immunoprecipitation and immunoblotted as indicated. Experiments with the various constructs were performed 2–3 times, and representative data are shown here.

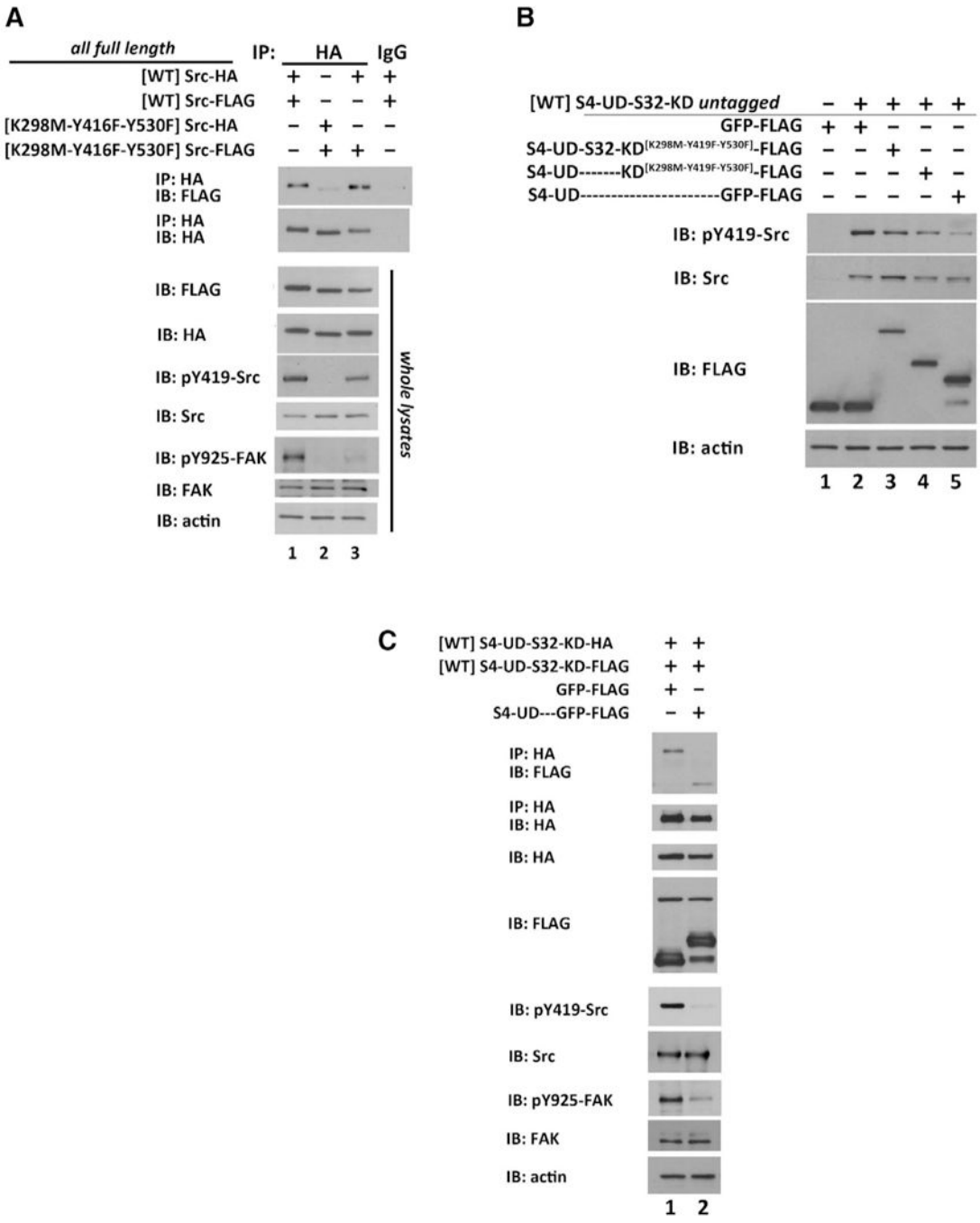

Figure 7. Interfering with Src Dimerization Disrupts Signaling

(A) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies. Immunoblots of whole-cell lysates were also performed as indicated.

(B) SYF cells were transfected with the indicated constructs and cell lysates immunoblotted as indicated.

(C) SYF cells were transfected with the indicated constructs. HA-tagged proteins were immunoprecipitated from cell lysates and immunoblotted with the indicated antibodies.

Immunoblots of whole-cell lysates were also performed as indicated. Experiments were performed twice, and representative data are shown here.